Optimizing hydrogen and volatile fatty acid production through dark fermentation of food waste: Exploring synergies in microbial consortium, pH modulation, and bioaugmentation strategies

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Food waste (FW) management has become a critical issue due to accelerating global growth, inefficient waste management infrastructure, and lack of awareness among the population. Therefore, effective, and sustainable waste management strategies have become imperative, where adverse environmental and health effects are minimized or eliminated, economically feasible, and can also produce value-added products. Traditional methods often need to be revised to address the issue's magnitude. Biological routes, like dark fermentation, emerge as promising strategies for efficient and eco-friendly treatment. Dark fermentation is a process where microbial consortia, like Clostridium, transform organic matter into valuable resources, mainly hydrogen (H_2) and volatile fatty acids (VFAs). H_2 can be used as an energy vector due to its high calorific value (141.7 MJ/kg) and low combustion residue, while VFAs have many applications, such as additives in animal feed, chemical synthesis precursors, or pollutant degradation in contaminated soils, among others. In dark fermentation processes, acid lactic bacteria, like *Lactobacillus*, can proliferate, and it supposes one of the main drawbacks and challenges of the system, because it can compete for substrate and produce lactic acid that will decrease H_2 and VFAs production efficiency. Strategies to control and optimize microbial diversity and activity are essential for enhancing the performance of dark fermentation. Recent research points to the possibility of synergistic activity between acidolactic and acidogenic bacteria to optimize the production of H_2 and VFAs, where both population ratio and the type of substrate are the main roles (Moreno-Andrade et al., 2023). This study aims to look for synergies between different bacteria of the *Lactobacillus* and *Clostridium* genera, studying the ratio between strains that constitute the microbial consortia to find the optimal conditions for H_2 and VFAs production using food waste as substrate. In addition, the effect of the operating pH and the application as a bioaugmentation strategy will be investigated.

Four strains have been tested for hydrogen production: 2 strains of *Clostridium* genus (*C*. beijerinckii ATCC 10132 and *C*. butyricum ATCC 19398) and 2 strains of *Lactobacillus* genus (*Lactobacilus plantarum* ATCC 8014 and *L. pentosus,* obtained and isolated from a hydrogen production reactor). All individual strains have been inoculated and incubated in serologic bottles and sterile atmosphere to a culture medium adapted to the growth and survival needs of the strains (ATCC Medium: 2107). Food waste characterization consisted of total solids (TS) and volatile solids (VS) analysis, chemical oxygen demand (COD), total carbohydrates content by Dubois method (Dubois et al., 1956) and pH measurement. Dark fermentation batch experiments comprised 120 mL serological bottles with 80 mL of final working volume. The reaction volume consists of food waste to a final concentration of 10 g/L in VS content, phosphate buffer solution pH 7.4 to reach a concentration from 0.005 M to 0.1 M, depending on the experiment, L-Cysteine at a concentration of 0.5 g/L to consume the dissolved oxygen, resazurin indicator to control the dissolved oxygen content and distilled water to a volume of 81 mL. Then, the reactors are sealed with a rubber septum and a metal seal and then the reactor head volume is gassed with nitrogen to achieve an inert atmosphere. The sealed and sterile bottles are autoclaved at 121ºC for 1 hour on sterile food waste experiments. After that, the cooled-down reactors are inoculated with the corresponding strain combination at a ratio of 10%v/v until a volume of 90 mL with the desired final optical density. Finally, 10 mL of the well-mixed medium is removed for further analysis of the reaction initial time and stored at -20°C. The reactors with the working volume are placed in the incubator at 37°C and 150 rpm. Gas phase analysis will be performed periodically with a gas syringe connected to a 3-way valve to determine the total volume produced and a gas chromatograph to determine its composition. When the reaction is stopped, and the bottles are opened, the liquid effluent is analyzed for carbohydrates by the Dubois method, VFAs, and lactate by HPLC and pH measurement.

The experimental set of 30 dark fermentation batch reactors was set up using sterilized food waste and 0.005 M buffer concentration with 15 combinations of the tested strains, covering the full spectrum of possible combinations. Figure 1A shows the tested combinations on accumulated hydrogen production per gram of VS_{added}. There is a lack of H_2 in all experiments in the absence of *C. beijerinckii*. The optimal H_2 production occurs in the conditions of experiment N (consisting of 61%$_{v/v}$ of *C. beijerinckii* and 13% of each other's), followed by C (100% $_{v/v}$ *C. beijerinckii*) and K (25% $_{v/v}$ of every strain), so it can be concluded that *C. beijerinckii* is a key strain in H_2 production. It is also enhanced by the minor presence of *C. butyricum*, *L. plantarum* and *L. pentosus* strains, but the positive effect on production is no longer observed when they are found in excess. Experimental conditions of set N were selected as optimal conditions. As verification, the experiment was carried out again at the same conditions and with higher pH buffer of 0.1 M, to evaluate its effect. It showed that the production in the experiment with 0.1 M buffer concentration had a 49% higher hydrogen production than the one with 0.005 M concentration, being the final pH of each reactor effluent 6.8 and 3.9 respectively, so it could be proved that an effective pH control is a key role in dark fermentation. After that, it was tested the effect of optimal conditions of strains combination and pH buffer concentration with non-sterile FW. Results are shown in Figure 1B, showing 14% lower production in terms of H_2/g_{VS}. It can be a consequence of thermal hydrolysis during food waste sterilization, which increases the availability of the organic matter to the inoculum.

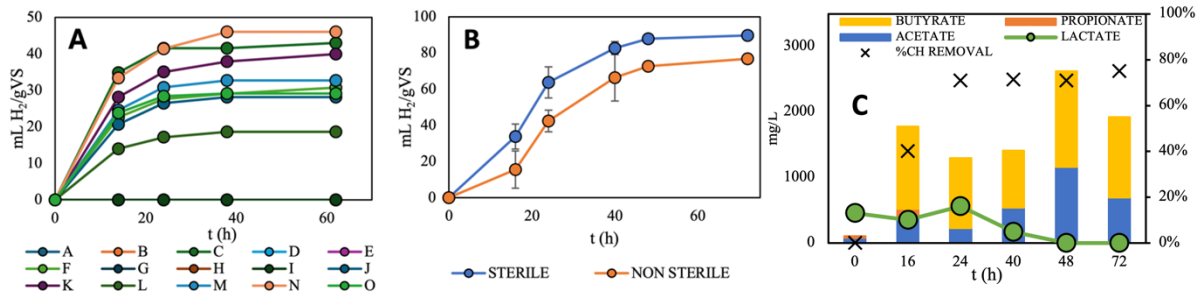

Figure 1. A) Hydrogen production from different experiments, B) Hydrogen production with sterile and non-sterile substrate, C) Metabolites production from sterile substrate.

These experiments were carried out with sacrifices to understand the changes that take place in the reaction medium during the reaction. The results on VFAs, lactate concentration, and % carbohydrate removal at each stage with sterile FW are shown in Figure 1C. Sterile conditions promote carbohydrates consumption over non-sterile, but this one produces more VFAs, especially acetic acid (2.1 g/L). Both consumes completely lactic acid content, highlighting the production under sterile conditions between time 0 and 24 h followed by total consumption at the end time, showing evidence of the synergy between acidolactic and acidogenic bacteria in the tested conditions. DNA was extracted from each analysis point of both sterile and non-sterile experiments, aiming to analyze the effluent content by metagenomic sequencing to know the taxonomy and evolution during reaction and understand the behavior of the reactors.

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